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Problem 2

FINAL REPORT

PRODUCTION OF SOUTHERN PINE BEETLES FREE OF MICROBES IN THE GUT FOR USE IN STUDIES OF SOURCE OF PHEROMONES

A cooperative agreement with
Louisiana State University at Alexandria
and
Southern Forest Experiment Station

Cooperative Agreement # 19-209

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Introduction

The association of microbes with various insects is well documented. Many have reviewed the microbes with an eye toward natural control of insect populations, but seldom is the relationship of the microbe and its host insect delved into very deeply. Many bacteria, yeast, and fungi are associated with Southern Pine Beetle, Dendroctonus frontalis, and their role in the insect's life cycle is currently being investigated. One aspect of this relationship is the role of associated microbes in the production of pheromones eliciting behavioral responses in the Southern Pine Beetle (SPB).

Studies at pheromone production in scolytids and SPB in particular have not shown the source of compounds. There are indications that micro-organisms can play a significant role in converting tree terpenes such as α-pinene into cis and trans-verbenol, verbenone, and other compounds.

Recent studies have indicated that Bacillus cereus from the SPB is capable of pheromone production and that a mycangial fungus had similar capabilities. Barras and Marler showed that B. cereus and other bacteria are common inhabitants of the gut and galleries of the SPB. Because the SPB has not been shown to produce pheromones in cells associated with the gut tissue, the possibility exists that the insect per se, does not produce most or all of the behavioral chemicals claimed to originate with the insect.

Objectives

The two objectives of the project were: 1) to produce SPB free of internal and external microbes; 2) to cooperate with Dr. J. M. Brand and Dr. Robert Bridges in the following aspects: to supply Dr. Brand and Dr. Bridges with microbe-free pine beetles, and to isolate and identify microbes (mainly bacteria and yeast) from the gut and/or galleries of SPB for use in studies on the influence of microorganisms on pheromonal regime of SPB.

PRODUCTION OF MICROBIAL FREE SPB

Pine bolts were field collected and utilized when most of the SPB reached the pupae stage. The pupae from infested bolts were removed and placed on lightly moistened filter paper (Whatman No. 1). Care must be taken to keep moisture at a minimum or mortality of pupae increases drastically.

Surface sterilization with modified White's solution (Table 1) was performed using the method worked out by Stanley J. Barras (S.F.E.S., Pineville, La.). Twenty (20) pupae were placed in a washing basket and washed for one (1) minute in sterile distilled water. From there, the pupae were treated four (4) minutes in modified White's solution, then into two (2) distilled (sterile) water rinses for one (1) minute each. The pupae were aseptically transferred to sterile filter paper to remove excess water and then placed on 3.0% water agar (3.0 gms agar/100 ml H_2O) plates. Plates were incubated at 30° C until the pupae molted.

After molting into adults, the surface sterile beetles were placed in an antibiotic artificial wood diet. This wood diet (Table II) consisted of Alphacel™, ground freeze-dried pine phloem, wheat germ, agar, ascorbic acid, vitamin mix (Nut. Biochem. Co.), and water. The antimicrobial agents were added after autoclaving, when the diet reached a temperature of about 60° C. The wood diet was "spooned" into 50 mm glass petri plates and allowed to harden. Plastic petri dishes should be avoided because the adult beetle will chew through the plastic plates. Plugs were cut out of the wood diet and one SPB was placed in each "well". The wells were capped over tightly and the SPB were incubated at 30° C for seven (7) days. Vigorous SPB were able to eat their way to the surface in two days while less vigorous adults required up to seven (7) days for emergence.

Statistically, approximately sixty (60) percent of the surface sterilized SPB pupae molted into adults. Only about forty-two (42) percent of these surface sterile adults were able to emerge from the diet. The emergence percentage drops when the number of beetles per plate is increased. It is recommended that no more than four (4) beetles be placed in a 50 mm plate.

TESTING FOR THE CONTAMINATION OF SPB

INTERNAL AND EXTERNAL STERILE SPB

As the SPB emerged from the wood diet, they were crushed and streaked on trypic soytone (tryp) and potato dextrose (pda) agars. The procedure used was as follows: A hanging drop slide was dipped in 95% alcohol and flamed. A sterile 100 mm petri plate lid served as a cover for the slide. Two to three drops of sterile physiological saline were placed on the slide. The beetle was positioned on the slide with forceps which were soaked in 70% alcohol. The initial crush was done with the forceps to prevent the beetle from crawling off the slide. A spatula, flamed in 95% alcohol was used to finish crushing the beetle. The crushed beetle was streaked on tryp and pda agar plates.

Of the sixty-seven beetles tested only two beetles were contaminated, a contamination rate of 3.0% Table III. Contamination of the two beetles were isolated and various biochemical tests were also performed. Some of the bacteria were found to be typical of those seen in normal SPB, while the others were air-contaminants.

STERILE SPB PLACED ON NON-ANTIBIOTIC TREATED WOOD DIET

Beetles which had eaten through the wood within the two days were placed on top of the wood diet which contained no antibiotics. The purpose of this was to show that any bacteria found in the beetles' gut were eliminated rather than suppressed. The beetles were allowed to eat the non-antibiotic wood for one week in the 30° incubator. The SPB were crushed and streaked in the same manner as described in the section above on tryp and pda agar.

Six beetles were sampled in this manner with no growth of bacteria being observed. Therefore, the bacteria were not just suppressed but totally eliminated from the beetle with the use of the antibiotics in the wood diet.

SPB THAT DID NOT EMERGE

When beetles did not emerge from the wood diet, the wood diet was separated with a sterile spatula until they were found. Every beetle found was dead. However, the gut and the body were separated anyway and placed in trypic soytone broth. After one week only two beetles showed any microbial growth. Both were from the gut of the beetles.

Of the twelve beetles dissected only two showed any contamination: 17% contamination rate. However, it should be remembered that beetles were dead before being dissected. The amount of antibiotic wood diet which some of the beetles ingested could have been minimal.

GALLERY CULTURES

Sixteen cultures have been isolated from a gallery of SPB. They were taken from five positions along the gallery: head, middle, egg niche, young larval gallery, and the frass of the rear. Various biochemical tests and stains were performed on these cultures. These cultures served as a comparison standard for bacteria isolated from contaminated beetles that had undergone sterilization.

CONCLUSION

The production of SPB free of internal and external microbes can take as little as five days. The contamination experienced when producing these sterile beetles have been minimal. Eighty-five adult beetles were tested for contamination by three different methods. The result of this testing is shown in Table II. Another ninety-five sterile SPB have been sent to Dr. Robert Bridges, the station's designated representative. Studies into phermone production by the beetle itself is currently being conducted by Dr. Bridges (S.F.E.S., Pineville, La.).

TABLE I

Modified White's Solution

$HgCl_2$	1.0 gm
NaCl	6.5 gm
HCl	1.25 ml
Ethanol (95%)	2.50 ml
H_2O (sterile)	750 ml

TABLE II

Sterilization Diet

Alphacel™	25 gm
Pine Phloem (freeze-dried)	25 gm
Kretchner's Wheatgerm	30 gm
Agar	7 gm
Ascorbic Acid	1.5 gm
Vitamin Mix (N.B.C.)	5.0 gm
Water	200 ml
Sorbic Acid (20% in EtOH)	2.5 ml*
Gentimycin Sulfate (43.8 mg/ml)	1.0 ml*
Lotrimin™ (10 mg/ml)	0.75 ml*

*Add to diet after autoclaving

TABLE III

Contamination Rate of the SPB

Type of SPB Tested	No. of SPB Tested	No. of SPB Contaminated	Percent Contamination
Internal and External Sterile SPB	67	2	3.0
Sterile SPB Placed on non-antibiotic treated wood diet	6	0	0
SPB that did not emerge	12	2	16.6
TOTAL	85	4	4.7